methionine. As shown in table 1, glutamic and aspartic acids were high in abundance in DHA-I and comprised more than 20% of the total residues. A high content of aromatic acids, tyrosine and phenylalanine, was also observed. No half-cystine, methionine or histidine was detected. Lack of half-cystine indicates that the subunits of DHA-I are held together by a non-covalent bond, as has been shown in many hemagglutinins from various sources[5].

Table 2 shows the results of an inhibition test for hemagglutionation against rabbit erythrocytes by simple sugars. D-Galactose and lactose among the sugars tested showed strong inhibitory activity. The activity was also inhibited by D-galactosamine, N-acetyl-D-galactosamine, N-acetyl-D-galactosamine, and D-galacturonic acid. D-Glucuronic acid, a well-known inhibitor for the aggregation factor of sponge cells, showed only weak inhibitory activity. None of other sugars tested showed any effect at a concentration of 100 mM.

A minor component(s), appearing in the fast-eluting protein peak from a Sephadex G-50 column, was found to contain few contaminants in analytical disc gel electrophoresis. Differing from DHA-I, it reacted preferably with rabbit erythrocytes showing a minimum active concentration of 1.2 µg protein/ml but not with human AB0 erythrocytes even at a concentration of 150 µg protein/ml. Although hemagglutinating activity of the crude extract was not affected by the addition of EDTA, as mentioned above, the activity of the minor component(s), comprising about 30% of the total activity, was lost in the presence of EDTA at a concentration as low as 5 mM. This discrepancy may be mainly due to the unreliability of the microtiter plate method used determination of hemagglutinating activity.

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Carbohydrase and esterase activity in the gut of larval *Callosobruchus maculatus*

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Summary. Carbohydrase activity has been demonstrated in homogenates of the alimentary tracts of late instar larvae of *C. maculatus*: β-D galactosidase, α-D glucosidase and N-acetyl β-D glucosaminidase activities were comparable and significantly greater than α-D galactosidase, β-D glucosidase and α-D mannosidase. The effects of pH and substrate concentration are reported. The presence and changes in pattern of non-specific esterase activity in larval and adult gut homogenates is also described.
Key words. Callosobruchus maculatus; digestive enzymes; glycosidases; esterases.

Callosobruchus maculatus is a major storage pest of the cowpea, *Vigna unguiculata*[1]. To date relatively little information is available concerning the physiology and biochemistry of digestion in these important pest insects. Podoler and Applebaum[2] isolated and partially characterized an α-amylase from a total larval extract of *C. chinensis* and more recently Gatehouse and Anstee[3] have reported the presence, and partially characterised, four carbohydrase enzymes in homogenates of the alimentary tract of adult male and female *C. maculatus*. The present study was carried out to extend further our knowledge of the digestive enzymes present in the adult insect and to provide information concerning the presence and nature of various carbohydrase and esterase activities in late larval guts of *C. maculatus*, as a basis for future physiological investigations on digestion.

Materials and methods. A culture of *Callosobruchus maculatus* originating from Campinas, Brazil, was reared and maintained on seeds of *Vigna unguiculata* at 28 ± 0.5 °C and 60% relative humidity. The photoperiod was arranged to give 12 h light and 12 h dark.

Preparation of gut homogenates: The alimentary tracts were removed by dissection from late instar larvae and sexually mature animals of both sexes which had previously been killed by decapitation. Homogenization was carried out in cold (ca. 4 °C) distilled water using a Potter-Elvehjem homogenizer with a teflon pestle (clearance 0.1–0.15 mm) with 20 passes of the plunger at 1000 rev/min; the homogenization tube was surrounded by ice throughout this procedure. The resulting homogenate was then centrifuged at 9000 × g for 5 min at 4 °C in a Haematocrit centrifuge and the supernatant retained for enzyme assay. All homogenates were freshly prepared.

The activities of α-D and β-D glucosidase, α-D and β-D galactosidase, α-D-mannosidase and N-acetyl β-D-glucosaminidase were determined by the estimation of p-nitrophenol liberated by hydrolysis of the corresponding p-nitrophenyl glycoside as described previously[3,4]. The composition of the reaction mixtures was as follows:

Aryl α-D glucosidase: 200 µl 0.1 M McIlvane's citrate-phosphate buffer[5], pH 5.2, 50 µl 51.2 mM p-nitrophenyl α-D-glucopyranoside (to give a final concentration of 9.5 mM) and 20 µl homogenate were incubated at 37 °C for 15 min in sealed Eppendorf tubes, unless otherwise stated in the text. The reaction was terminated by the addition of 1 ml 50 mM NaOH and the absorbance read at 405 nm.

Aryl β-D glucosidase: as for aryl α-D glucosidase except for the use of the substrate p-nitrophenyl β-D glucopyranoside.

Aryl α-D galactosidase: as for aryl α-D glucosidase except for the use of the substrate p-nitrophenyl α-D galactopyranoside (35 mM: to give a final concentration of 6.5 mM).

Aryl β-D galactosidase: as for aryl α-D galactosidase except for the use of the substrate p-nitrophenyl β-D galactopyranoside.

Aryl α-D mannosidase: as for aryl α-D glucosidase except for the use of the substrate p-nitrophenyl α-D mannoside (25 mM: to give a final concentration of 4.6 mM) and pH 4.6.

Aryl N-acetyl β-D glucosaminidase: as for aryl α-D mannosidase except for the use of the substrate p-nitrophenyl N-acetyl β-D glucosaminide.

Electrophoresis: Thin layer non-SDS polyacrylamide gel electrophoresis was carried out using the method of Gabriel[6]; 7.5% acrylamide gels were used. Aliquots of the homogenate, prepared as described above, were applied to the gel and following electrophoretic separation the gels were stained for nonspecific esterase or β-galactosidase activity.

Esterase activity: Following electrophoresis the gel was incubated for 5 min in a substrate solution consisting of 1.6 ml of 1% α-naphthyl acetate in ethanol in a final volume of 100 ml 0.2 M acetate buffer, pH 5.0. The staining dye was Fast Blue B[7].

β-Galactosidase activity: Following electrophoresis the gel was incubated in McIlvane's buffer, pH 5.0, for 15 min followed by 30 min in a 1 mM solution of 4-methylumbelliferyl-β-D galactoside in McIlvane's buffer, pH 5.0. The gel was then rinsed for 1 min in 50 mM NaOH prior to being viewed under an UV light source.

Protein estimations were carried out using the method of Lowry *et al.*[8] with bovine serum albumin Fraction V (Sigma Chemical Co.) as standard.

All solutions were prepared in glass distilled water, buffer components and reagents were obtained from BDH Chemical Ltd., Poole, Dorset, and were AnalaR grade or the best commercially

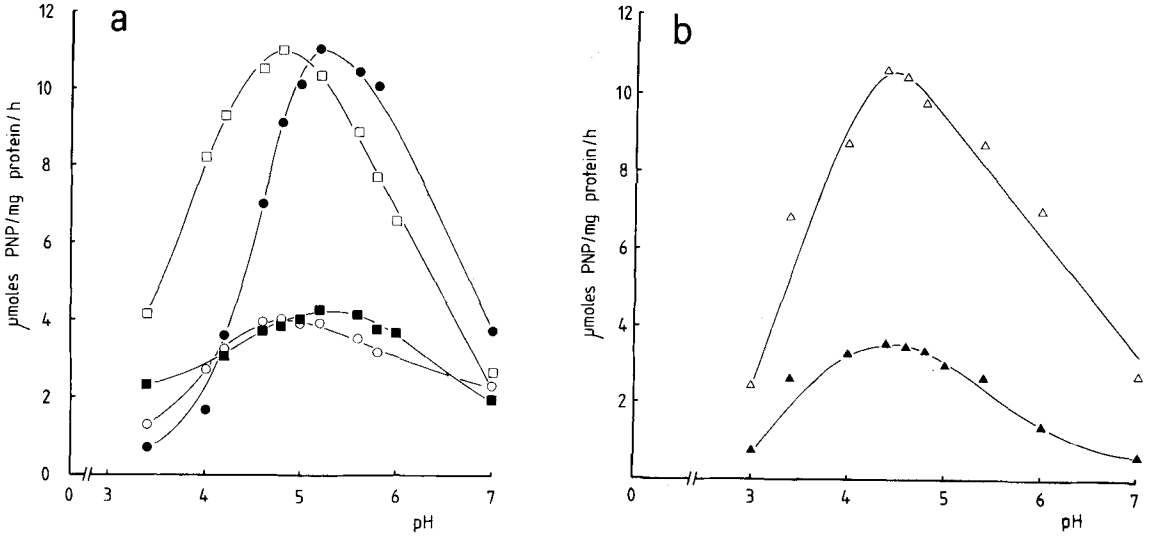

Figure 1. pH profiles of glycosidases present in the larval gut of *C. maculatus*.
a ●, α-D glucosidase activity; ○, β-D glucosidase activity; ■, α-D galactosidase activity; □, β-D galactosidase activity.
b △, N-acetyl β-D glucosaminidase activity; ▲, α-D mannosidase activity.

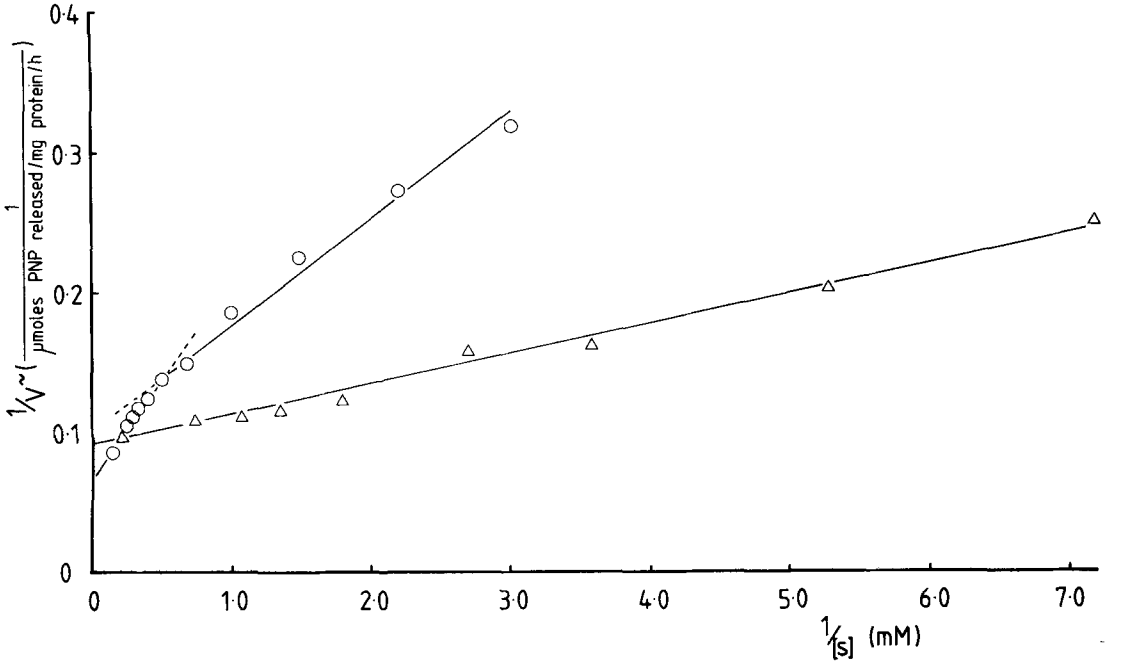

Figure 2. Lineweaver-Burk plots of two of the larval gut glycosidases of *C. maculatus* showing a linear plot (△, N-acetyl β-D-glucosaminidase activity) and a biphasic plot (○, β-D galactosidase activity).

available. The enzyme substrates α- and β-D-glucopyranoside, α- and β-D-galactopyranoside, p-nitrophenyl-α-D mannopyranoside, p-nitrophenyl-N-acetyl-β-D-glucosaminide, α-naphthyl acetate, and 4-methylumbelliferyl-β-D galactoside were obtained from Sigma London Chemical Company Ltd., Poole, Dorset, as was the enzyme inactivator L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK).

Results and discussion. The oligosaccharidases α-D and β-D glucosidase, α-D and β-D galactosidase, α-D mannosidase and N-acetyl β-D glucosaminidase were present in homogenates of the alimentary tracts of late instar larvae of *Callosobruchus maculatus*. The most active of these glycosidic enzymes were the β-D galactosidase (sp. act. ± SEM being 11.06 ± 0.17 μmoles p-nitrophenol (PNP) released/mg protein/h; n = 4), the α-D glucosidase (sp. act. ± SEM being 11.04 ± 0.65 μmoles PNP released/mg protein/h; n = 3) and N-acetyl β-D glucosaminidase (sp. act. ± SEM being 10.60 ± 0.87 μmoles PNP released/mg protein/h; n = 4). The activities of the α-D galactosidase (sp. act. ± SEM being 4.30 ± 0.47 μmoles PNP released/mg protein/h; n = 3), the β-D glucosidase (sp. act. ± SEM being 4.01 ± 0.36 μmoles PNP released/mg protein/h; n = 3) and the α-D mannosidase (sp. act. ± SEM being 3.51 ± 0.11 μmoles PNP released/mg protein/h; n = 3) were substantially lower. This distribution of glucosidase and galactosidase activity contrasts markedly with that reported previously[3] for the adult insect in which β-D galactosidase > α-D glucosidase > α-D galactosidase > β-D glucosidase. Indeed, the level of β-D glucosidase activity in the adult insect was reported to be so low that it was excluded from further studies. It is clear, therefore, that there has been a marked change in the relative activities of these enzymes at metamorphosis.

Apparent K_m and V_{max} values for glycosidases present in the larval gut of *C. maculatus*

Enzyme	Range of substrate concentrations (mM)	K_m (mM)	V_{max} μmoles PNP released/mg protein/h
α-D-glucosidase	0.19–0.95	0.92	8.60
		0.64	5.58
	1.27–6.33	2.74	18.07
		3.05	14.84
β-D-glucosidase	0.19–0.95	0.18	1.47
		0.13	1.21
	1.27–9.5	3.11	4.98
		2.86	3.77
α-D-galactosidase	0.33–1.5	0.27	1.73
		0.30	2.99
	2.0 –6.48	1.36	2.50
		1.05	3.88
β-D-galactosidase	0.33–1.5	0.60	8.72
		0.56	5.89
	2.0 –6.48	2.09	14.69
		1.72	9.40
α-mannosidase	0.14–0.37	0.11	1.67
	0.55–4.63	0.71	3.56
N-acetyl β-D-glucosaminidase	0.09–4.63	0.23	11.02

Where two values are given these represent two independent experiments.

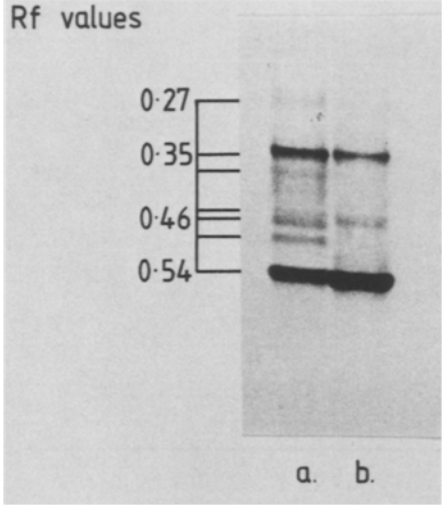

Figure 3. Polyacrylamide gel electrophoresis (on 7.5% gel slab) of gut homogenates of *C. maculatus* stained for non-specific esterase activity; Track a, larval gut homogenate; track b, adult gut homogenate.

The effects of pH on the activities of these carbohydrases was studied using McIlvane's buffer to produce a stable range of pH from 3.0 to 7.0. The results obtained are shown in figure 1. Maximal activity was observed at pH 5.2 for α-Dglucosidase and α-D galactosidase and at pH 4.8 for β-D glucosidase and β-D galactosidase. These values agree well with those reported for digestive enzymes from a variety of different insect species[9–13] including adult *C. maculatus*[3]. In the case of N-acetyl β-D glucosaminidase and α-D mannosidase maximal activities were observed at pH 4.6. Again, these values are in good agreement with those reported elsewhere[14].

The effect of substrate concentration on the activities of these six glycosidases was determined under optimal pH conditions. Care was taken to ensure that substrate availability did not become rate-limiting during the course of the reactions. The apparent Michaelis constant (K_m) and V_{max} were calculated from Lineweaver-Burk plots of the data for each enzyme (table 1). The affinities of these enzymes for their respective substrates compare reasonably well with values reported elsewhere in various other insect species[3, 9, 11, 13]. The small differences noted between independent experiments are probably not significant. In the present study only N-acetyl β-D glucosaminidase showed simple Michaelis-Menton kinetics (figure 2). The other carbohydrases exhibited biphasic Lineweaver-Burk plots similar to those reported for the α-D glucosidase in the gut of adult *C. maculatus*[3]. Biphasic plots have also been reported for β-D glucosidase and β-D galactosidase from the gut of *Locusta migratoria*[9]. However, in adult *C. maculatus* such plots of α-D and β-D galactosidase activity were linear. The explanation of such biphasic plots is uncertain. It may be that the homogenates contained a number of different enzymes or isoenzymes, each with differing affinities for the substrate. Some support for this comes from the fact that three β-D glucosidase components have been reported in the crop of *Locusta*[15] and biphasic Lineweaver-Burk plots have also been reported for this enzyme in this species[9]. In the present study, electrophoresis of gut homogenates from the larvae of *C. maculatus* followed by incubation of the gel in the presence of the β-D galactosidase substrate, 4-methylumbelliferyl-β-D-galactoside, revealed three distinct bands with Rf values of 0.29, 0.42 and 0.48, which also tends to support this proposal. However the fact that three β-D galactosidase bands were also separable in homogenates of adult guts (Rf values being 0.45, 0.50 and 0.53) whereas this enzyme showed linear Lineweaver-Burk plots[3] argues caution in accepting this view. An alternative explanation is that there is some change in the kinetic parameters of the system in response to different concentrations of the substrate. The substrate may be acting not only on the enzyme as a substrate but also as a regulator which controls the binding of substrate and rate of decomposition of the enzyme-substrate complex[16]. Clearly further studies are necessary before the significance of such bi-phasic plots can be explained in relation to the physiology of digestion in *Callosobruchus maculatus*. Comparison of the Rf values for adult and larval β-D galactosidase activity suggest that whilst two bands may be common to both stages in the life cycle (i.e. R_f 0.42 and 0.48 in the larvae and R_f 0.45 and 0.50 in the adult) the third is clearly not.

Enzymes capable of splitting the β-linkages of N-acetylglucosamine polymers have been reported in several insect tissues, particularly in the gut, where they may have a digestive function[17]. Glycoproteins in plants are known to contain carbohydrate side-chains consisting of N-acetylglucosamine and mannose residues. It is perhaps significant, therefore, that N-acetyl β-D glucosaminidase and α-D mannosidase activity was present in *C. maculatus* larvae which feed on the seeds of the cowpea, *Vigna unguiculata*, which contain large amounts of such glycoproteins[18]. Similar enzymes have been reported in the alimentary tracts of other insect species. For example, in the crop of *Schistocerca gregaria*[19] and the midgut of *Stomoxys calcitrans*[14].

Esterases are ubiquitous non-specific enzymes that catalyse the hydrolysis of ester linkages. Such enzymes have been implicated in a variety of functions in insects. These include lipid digestion, the general metabolism and mobilisation of fats, energy-related fat catabolism in muscles and the synthesis and transport of cuticular wax[20, 21]. In addition, a large number of xenobiotics to which insects are exposed are metabolised by esterases. For example, the presence of carboxyl ester groups in malathion and other insecticides makes these compounds vulnerable to hydrolysis by carboxylesterases[22]. In the present study thin layer polyacrylamide gel electrophoresis revealed the presence of a number of bands exhibiting non-specific esterase activity in homogenates of both larval and adult guts (figure 3). Seven distinct esterase bands were present in the larval preparation (Rf values being 0.27, 0.35, 0.38, 0.45, 0.46, 0.48, 0.54) whilst only four bands were detected in the adult (R_f values being 0.35, 0.38, 0.45, 0.55). Similar changes in gut esterases have been reported during the life cycle of other insects. Clements[20] reported a marked fall in the number of esterases in the pupal stage, as compared to the larval stages, of *Pieris brassicae*. Whilst there was a recovery of most of the late instar larval esterases in the adult *Pieris*, not all were present in detectable amounts. Desnuelle and Savary[23] have pointed out that most of the substrates used for characterising esterase activity can be hydrolysed by proteolytic enzymes such as chymotrypsin and trypsin. Since recent studies have revealed the presence of a proteolytic enzyme in larval gut homogenates of *C. maculatus* which was relatively insensitive to the Kunitz soyabean trypsin inhibitor (Gatehouse, unpublished data) it was decided to examine the effect of the chymotrypsin inhibitor TPCK on the activities of the various esterases present in adult and larval guts of *C. maculatus*. In both stages of the life cycle this agent was without significant effect on esterase activity. This clearly confirms that the bands are almost certainly esterases and not merely side-effects of proteolytic enzymes. Further studies are in progress to determine the inhibition characteristics of these esterases.

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Malarial parasites complete sporogony in axenic mosquitoes[1]

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Summary. To obtain sporogonic stages of malaria free from microbial contaminants for in vitro studies, *Anopheles stephensi* were reared under sterile conditions using a mosquito cell line as larval food. The adult females, kept in sterile humidified containers and allowed to engorge on parasitemic hamsters, supported the sporogonic development of the rodent malarial parasite *Plasmodium berghei*. In 10 experiments, the proportion of infected mosquitoes varied from 0 to 92%, and the geometric mean number of oocysts per female mosquito from 2.5 to 58.6, with a range of 1 to 548. The average number of salivary gland sporozoites per infected mosquito was determined by direct sporozoite counts in the pooled homogenate of the thoraces of all female mosquitoes. In five experiments, it varied from 2.7×10^3 to 9.0×10^3. The sterile sporozoites, harvested on day 19 or 20 after the infective blood meal, were as infective for rodents as nonsterile ones.

Key words. Anopheles stephensi; Plasmodium berghei; axenic mosquitoes; transmission of malaria; mosquito cell culture.

In the mosquito, the malarial parasite undergoes a series of changes that begins with the formation of gametes and culminates in the production of sporozoites infective for the vertebrate host. These events have not been analyzed experimentally in detail because of the lack of in vitro systems that allow the formation and manipulation of the oocyst and the developing sporozoites within.

Several researchers have reared mosquitoes under sterile conditions[2], but the resulting adults are unable to support complete development of mosquito-born parasites[3]. We have found that axenic *Anopheles stephensi* can transmit rodent malaria caused by *Plasmodium berghei*[4], and we now demonstrate for the first time that malarial parasites complete sporogonic development in axenic mosquitoes and differentiate into mature sporozoites.